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(54) Title: PRODUCTION OF A MULTIMERIC PROTEIN BY CELL FUSION METHOD

(57) Abstract

The present invention features a method of producing a multimeric protein from a hybrid cell formed from the fusion of two or more cells, each of which cell is engineered to express one component of the multimeric protein, as well as a method for screening for successful fusion of the cells to produce a desired hybrid cell. The methods of the invention are widely applicable to the production of proteins having two or more components.

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PRODUCTION OF A MULTIMERIC PROTEIN BY CELL FUSION METHOD

FIELD OF THE INVENTION

This invention relates generally to methods for use in gene expression and cell fusion techniques, particularly in the production of multi-component proteins.

BACKGROUND OF THE INVENTION

Recombinant DNA techniques have been used for production of heterologous proteins in transformed host cells. Generally, the produced proteins are composed of a single amino acid chain or two chains cleaved from a single polypeptide chain. More recently, multichain proteins such as antibodies have been produced by transforming a single host cell with DNA sequences encoding each of the polypeptide chains and expressing the polypeptide chains in the transformed host cell (U.S. Patent No. 4,816,397).

The basic immunoglobulin (Ig) structural unit in vertebrate systems is composed of two identical "light" polypeptide chains (approximately 23 kDa), and two identical "heavy" chains (approximately 53 to 70 kDa). The four chains are joined by disulfide bonds in a "Y" configuration, and the "tail" portions of the two heavy chains are bound by covalent disulfide linkages when the immunoglobulins are generated either by hybridomas or by B cells.

A schematic of the general antibody structure is shown in Fig. 1. The light and heavy chains are each composed of a variable region at the N-terminal end, and a constant region at the C-terminal end. In the light chain, the variable region (termed "V_LJ_L") is the product of the recombination of a V_L gene to a J_L gene. In the heavy chain, the variable region ($V_HD_HJ_H$) is the product of recombination of first a D_H and a J_H gene, followed by a D_HJ_H to V_H recombination. The V_LJ_L and $V_HD_HJ_H$ regions of the light and heavy chains, respectively, are associated at the tips of the Y to form the antibody's antigen binding domain and together determine antigen binding specificity.

The (C_H) region defines the antibody's isotype, i.e., its class or subclass. Antibodies of different isotypes differ significantly in their effector functions, such as the ability to activate complement, bind to specific receptors (Fc receptors) present on a wide

variety of cell types, cross mucosal and placental barriers, and form polymers of the basic four-chain IgG molecule.

Antibodies are categorized into "classes" according to the C_H type utilized in the immunoglobulin molecule (IgM, IgG, IgD, IgE, or IgA). There are at least five types of C_H genes ($C\mu$, $C\gamma$, $C\delta$, $C\epsilon$, and $C\alpha$), and some species (including humans) have multiple C_H subtypes (e.g., $C\gamma_1$, $C\gamma_2$, $C\gamma_3$, and $C\gamma_4$ in humans). There are a total of nine C_H genes in the haploid genome of humans, eight in mouse and rat, and several fewer in many other species. In contrast, there are normally only two types of light chain constant regions (C_L), kappa (κ) and lambda (λ), and only one of these constant regions is present in a single light chain protein (i.e., there is only one possible light chain constant region for every V_LJ_L produced). Each heavy chain class can be associated with either of the light chain classes (e.g., a $C_H\gamma$ region can be present in the same antibody as either a κ or λ light chain).

A process for the immortalization of B cell clones producing antibodies of a single specificity has been developed involving fusing B cells from the spleen of an immunized mouse with immortal myeloma cells. Single clones of fused cells secreting the desired antibody could then be isolated by drug selection followed by immunoassay. These cells were given the name "hybridoma" and their antibody products termed "monoclonal antibodies."

The use of monoclonal antibodies as therapeutic agents for human disease requires the ability to produce large quantities of the desired antibody. One approach to increased production was simply to scale up the culture of hybridoma cells. Although this approach is useful, it is limited to production of that antibody originally isolated from the mouse. In the case where a hybridoma cell produces a high affinity monoclonal antibody with the desired biological activity, but has a low production rate, the gene encoding the antibody can be isolated and transferred to a different cell with a high production rate.

In some cases it is desirable to retain the specificity of the original monoclonal antibody while altering some of its other properties. For example, a problem with using murine antibodies directly for human therapy is that antibodies produced in murine systems may be recognized as "foreign" proteins by the human immune system, eliciting a response against the antibodies. A human anti-murine antibody (HAMA) response results in antibody neutralization and clearance and/or potentially serious side-effects associated

with the anti-antibody immune response. Such murine-derived antibodies thus have limited therapeutic value.

One approach to reducing the immunogenicity of murine antibodies is to replace the constant domains of the heavy and light chains with the corresponding human constant domains, thus generating human-murine chimeric antibodies. Chimeric antibodies are generally produced by cloning the antibody variable regions and/or constant regions, combining the cloned sequences into a single construct encoding all or a portion of a functional chimeric antibody having the desired variable and constant regions, introducing the construct into a cell capable of expressing antibodies, and selecting cells that stably express the chimeric antibody. Examples of methods using recombinant DNA techniques to produce chimeric antibodies are described in PCT Publication No. WO 86/01533 (Neuberger et al.), and in U.S. Patent Nos. 4,816,567 (Cabilly et al.) and 5,202,238 (Fell et al.).

In another approach, complementarity determining region (CDR)-grafted humanized antibodies have been constructed by transplanting the antigen binding site, rather than the entire variable domain, from a rodent antibody into a human antibody. Transplantation of the hypervariable regions of an antigen-specific mouse antibody into a human heavy chain gene has been shown to result in an antibody retaining antigen-specificity with greatly reduced immunogenicity in humans (Riechmann et al. (1988) Nature 332:323-327; Caron et al. (1992) J. Exp. Med 176:1191-1195).

Another approach in the production of human antibodies has been the generation of human B cell hybridomas. Applications of human B cell hybridoma-produced monoclonal antibodies have promising potential in the treatment of cancer, microbial infections, B cell immunodeficiencies associated with abnormally low antibody production, and other diseases and disorders of the immune system. Obstacles remain in the development of such human monoclonal antibodies. For example, many human tumor antigens may not be immunogenic in humans and thus it may be difficult to isolate anti-tumor antigen antibody-producing human B cells for hybridoma fusion.

For a given disease indication, one antibody isotype is likely to be greatly preferred over another. The preferred isotype may vary from one indication to the next. For example, to treat cancer it may be desirable that the binding of an antibody to a tumor cell result in killing of a tumor cell. In this case, an IgG1 antibody, which mediates both

antibody-dependent cellular cytotoxicity and complement fixation, would be the antibody of choice. Alternatively, for treating an autoimmune disease, it may be important that the antibody only block binding of a ligand to a receptor and not cause cell killing. In this case, an IgG4 or IgG2 antibody would be preferred. Thus, even in a situation where a high affinity, antigen-specific, fully human antibody has been isolated, it may be desirable to re-engineer that antibody and express the new product in a different cell.

The growing use of phage display technology also points to a need for antibody engineering and expression methodologies. Phage display technology is used for producing libraries of antibody variable domains cloned into bacteria. This allows variable domains of desired specificity to be selected and manipulated *in vitro*. While bacteria offer a great advantage for selecting and producing antibody fragments, they are not capable of producing full-size intact antibodies in native configuration, and it is necessary to reconstitute fragments selected in bacteria into intact antibodies and express them in eucaryotic cells.

SUMMARY OF THE INVENTION

The present invention features a method of producing a multimeric protein from a hybrid cell formed from the fusion of two or more cells, each of which cell is engineered to express one component of the multimeric protein, as well as a method for screening for successful fusion of the cells to produce a desired hybrid cell. The methods of the invention are widely applicable to the production of proteins having two or more components.

In one specific application of the method of the invention, the multimeric protein is an antibody composed of antigen-specific heavy and light chains. DNA encoding the desired heavy chain (or a fragment of the heavy chain) is introduced into a first mammalian host cell, while DNA encoding the desired light chain (or a fragment of the light chain) is introduced into a second mammalian host cell. The first transformed host cell and the second transformed host cell are then combined by cell fusion to form a third cell. Prior to fusion of the first and second cells, the transformed cells may be selected for specifically desired characteristics, e.g., high levels of expression. After fusion, the resulting hybrid cell contains and expresses both the DNA encoding the desired heavy chain and the DNA encoding the desired light chain, resulting in production of the multimeric antibody.

In one aspect the invention features the multimeric protein produced by the method of the invention. In one embodiment, the invention includes an antibody produced by the method of the invention.

In another aspect the invention features a method for screening for successful fusion of a first cell containing a first nucleotide sequence encoding a desired antibody heavy chain and a second cell containing a second nucleotide sequence encoding a desired antibody light chain, the method comprising including a nucleotide sequence encoding a first marker gene in the first cell, including a nucleotide sequence encoding a second marker gene in the second cell, fusing the first and second cells to produce a fused cell and assaying for the presence of the first and second marker genes in the fused cell.

One advantage of the method of the invention is that cells expressing a single component of the final multi-component protein can be individually selected for one or more desired characteristics, such as a high rate of production.

Another advantage is that the method generates a cell which produces an antibody at a multiplication high rate through the fusion of two kinds of cells which are each selected prior to fusion for high production of the desired heavy or light chains.

Another advantage is that the final multi-component protein is not expressed until all the cells expressing the individual components of the multi-component protein are fused into a single hybrid cell.

Other aspects, features, and advantages of the invention will become apparent from the following detailed description, and the claims.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a schematic showing the basic immunoglobulin structure.

Figure 2 is a flow chart showing one embodiment of the method of invention when mammalian cells are separately transformed with the desired light and heavy chain DNA, then fused to form the hybrid cell expressing both chains.

Figure 3 illustrates a specific embodiment of the invention in which a mammalian cell expressing an irrelevant light chain is transformed with the desired heavy chain DNA, a second mammalian cell is transformed with the desired light chain DNA, and the desired hybrid cell formed from fusion of the transformed host cells is selected which expresses the desired antibody product.

Figure 4 is a schematic illustrating a specific embodiment of the invention in which DHFR CHO cells are independently transfected with (i) pManuGamma#6, a human heavy chain Ig construct and (ii) pManuKappa#14, a human light chain Ig construct. The independent cell lines are selected, amplified, fused, and selected to yield a hybrid cell containing the human heavy chain Ig construct and the human light chain Ig construct.

Figure 5 is a schematic diagram of a fusion method in accordance with the present invention demonstrating the use of HPRT and LacZ marker genes for the initial determination of the success of a fusion process.

Figure 6 is a schematic diagram of the IgK expression vector (pLS413) and the IgH expression vector (pLS421).

Figure 7 is a graph showing the results of an ELISA assay to determine the IL-8 binding affinity of antibodies produced by the cell fusion products of Example 2. Open circles, F-2 fusion clone; open squares, F-5 fusion clone; open triangles, F-13 fusion clone; open diamonds, F-15 fusion clone; closed bar, F-16 fusion clone; closed stars, F-17 fusion clone; closed circles, negative control human IgG antibody (no binding affinity for IL-8); and closed squares, D39.2 anti-Il-8 antibody (starting antibody).

Figure 8 is a schematic representation of a construct used for transfection of cells with heavy or light chain Ig cassettes and selectable marker cassettes.

Figure 9 is a restriction map of the human heavy chain construct H/Pur/pEE12.1 containing the puromycin resistance gene.

Figure 10 is a restriction map of the human kappa light chain construct K/Hyg/pEE12.1 containing the hygromycin resistance gene.

DETAILED DESCRIPTION

Before the methods and compositions of the present invention are described and disclosed it is to be understood that this invention is not limited to the particular methods and compositions described as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting since the scope of the present invention will be limited only by the appended claims.

It must be noted that as used in this specification and the appended claims, the singular forms "a", "an" and "the" include plural references unless the context clearly

dictates otherwise. Thus, for example, reference to "a DNA sequence" includes a plurality of DNA sequences and different types of DNA sequences.

Unless defined otherwise all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any materials or methods similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described. All publications mentioned herein are incorporated herein by reference for the purpose of describing and disclosing the particular information for which the publication was cited. The publications discussed above are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the inventor is not entitled to antedate such disclosure by virtue of prior invention.

Definitions

By the term "**nucleotide sequence**" is meant any DNA fragment of interest which may be introduced into a cell, including an intact gene or fragment of a gene. When the method of the invention is used to generate an antibody, the nucleotide sequence of interest will be all or part of either the constant region and/or variable region of the light or heavy chains, and may include all, part, or none of the regulatory nucleotide sequences that control expression of the light or heavy chain. The nucleotide sequence of interest for heavy chains includes but is not limited to all or a portion of the V, D, J, and switch regions (including intervening sequences) and flanking sequences. For light chains, the nucleotide sequence of interest includes but is not limited to the V and J regions, and flanking and intervening sequences. The nucleotide sequence may be a naturally occurring sequence, synthetic, or partially natural and partially synthetic. The sequence may also be a non-naturally occurring or modified naturally-occurring sequence. The DNA sequence includes sequences taken from different sources, e.g., different species. For example, when the method is used to produce an antibody, the DNA chain may encode a chimeric (for example, human-mouse) immunoglobulin chain, or it may be a CDR-grafted DNA sequence having a human immunoglobulin sequence with antigen-specific murine CDR sequences. The DNA of the nucleotide sequence may encode a fully human antibody. B-cells obtained from non-human animals immunized with an antigen and also hybridoma, trioma, and quadromas derived from such B-cells can also provide the nucleotide sequence

introduced into the host cells. B-cells and hybridomas producing any kind of monoclonal antibody may be used as a source of the nucleotide sequence, including cells producing, for example, fully mouse monoclonal antibodies, fully human monoclonal antibodies, CDR-grafted monoclonal antibodies, chimeric monoclonal antibodies, and $F(ab)_2$.

By the terms "**multi-component**", "**multichain**", or "**multimeric**" protein is meant a protein composed of two or more proteins or polypeptides. The method of the invention is useful for producing a multimeric protein by the fusion of two or more cells each expressing a single component of the multimeric protein. For example, in one embodiment the multi-component protein is an antibody generated from two heavy chains encoded by DNA transfected into a first cell and two light chains encoded by DNA transfected into a second cell, where the final multimeric antibody is produced by a hybrid cell formed from the fusion of the first and second cells. "Multi-component," "multichain," and "multimeric" protein is meant to include any heterodimeric or hetero-oligomeric protein (e.g., BMP2/BMP7 heterodimeric osteogenic protein, ICE (interleukin-1 converting protein), receptors of the nucleus (e.g., retinoid receptors), heterodimeric cell surface receptors (e.g., T cell receptors), integrins (e.g, cell adhesion molecules, β_1-integrins, (see, e.g., Hynes, 1987 Cell 48:549-554; Hynes 1992 Cell 60:11-25), tumor necrosis factor (TNF) receptor, and soluble and membrane-bound forms of class I and class II MHC (major histocompatibility complex proteins). Where the multimeric protein is a receptor, "multimeric protein" is meant to encompass soluble and membrane forms of the receptor.

By the term "**introducing**" a nucleotide sequence into a cell means inserting an exogenous piece of DNA into a cell, including but not limited to transfection or transduction with a vector, such that all or part of the exogenous nucleotide sequence is stably maintained in the cell, and the resulting transformed cell expresses the introduced nucleotide sequence.

By the term "**fusing**" or "**fusion**" of two or more cells is meant a method in which two or more cells are combined to form a single hybrid cell which contains all or part of at least the nucleic acid content of each individual cell. Fusion may be accomplished by any method of combining cells under fuseogenic conditions well known in the art (See, for example, Harlow & Lane (1988) in Antibodies, Cold Spring Harbor Press, New York).

Known methods for fusing cells includes by use with polyethylene glycol (PEG) or Sendai virus.

By the term "**hybrid cell**" is meant a cell formed by combining two or more cells, e.g., by fusion. In the method of the invention, hybrid cells are formed from the fusion of one or more transformed cells each expressing a single component of a multimeric protein.

The term "**irrelevant**" as in, e.g., " an irrelevant light chain" means a light chain which does not contribute to the binding of the antigen of interest and is not a component of the multimeric protein produced by the hybrid cell of the invention.

By the term "**desired**" component, e.g., desired heavy chain, or desired light chain, is meant an immunoglobulin chain which recognizes the antigen of interest.

Generation of a Hybrid Cell Producing a Heterologous Multimeric Protein

The present invention provides a method for generating a hybrid cell producing a multi-component protein from two or more transformed cells each of which cells produces a single component of the multimeric protein. This method features several important advantages relative to conventional methods for protein production. For example, the method of the present invention allows separately transformed cells to be individually selected for optimal expression of each component of the multi-component protein. This selection occurs prior to fusion of cells forming the hybrid cell and prior to production of the final multimeric protein. The method of the invention results in a final multi-component protein product which is not expressed until a single hybrid cell is produced from the fusion of each cell expressing a component of the final protein product.

Generally, when the multi-component protein to be produced is an antibody, the method of the invention involves generation of a cell expressing a desired heavy chain, generation of a cell expressing a desired light chain, and fusion of the two cells to form a hybrid cell expressing the final antibody protein (Fig. 2). Generation of a cell expressing the desired heavy chain involves the following steps: (1) identifying and cloning and/or synthesizing the gene, gene fragment, or nucleotide sequence encoding the variable segment or antigen-binding sequences of the heavy chain. The nucleotide sequence may be obtained from either a cDNA or genomic source, or synthesized *de novo*; (2) cloning the nucleotide sequence encoding the desired constant regions of the heavy chain; (3) ligating the variable region with the constant region so that the complete nucleotide sequence can be transcribed and translated to express the desired heavy chain polypeptide;

(4) ligating the construct into a vector containing a selectable marker and appropriate gene control regions; (5) amplifying the construct in bacteria; (6) introducing the vector into eukaryotic cells; (7) selecting the cells expressing the selectable marker; and (8) screening the cell supernatants or lysates for the expressed heavy chain. Similarly, a cell expressing a desired light chain construct is generated as outlined above.

Alternatively, the process of generating a cell expressing a desired heavy or light chain may involve (1) construction of a Ig chain DNA sequence containing (a) a signal sequence, (b) the gene, gene fragment, or nucleotide sequence encoding the variable region or antigen-binding sequences, and (c) the nucleotide sequence encoding the desired constant region of the Ig chain, followed by (2) PCR amplification of the Ig construction, (3) insertion of the construct into eukaryotic cells, (4) selecting the cells expressing the selectable marker, and (5) screening the cells for the expressed Ig chain. Optionally, the cells expressing the desired heavy chain or the desired light chain can be further selected for desirable characteristics, such as heavy or light chain production rate or level, ability of the expressed heavy or light chain to combine with another light or heavy chain, respectively, to provide an antibody having a desired antigen binding affinity, and/or other characteristics desirable for heavy or light chain production or function in an antibody.

Transformed cells expressing or capable of expressing the desired component of the multimeric protein are fused by methods known in the art to form a hybrid cell expressing the multimeric protein. When the multimeric protein is an antibody, the DNA sequences encoding the desired immunoglobulin may be composed entirely of sequences originating from a single species, e.g., fully human or fully murine, or may be contain sequences originating from more than one species, e.g., a human-mouse chimera or CDR-grafted humanized antibody. The hybrid cell produced antibody product may also contain a desired antigen binding site (variable region) linked to a desired constant region. Thus, a specifically designed antibody may be generated with a desired antigenicity combined with the desired isotype.

Prior art methods for independently expressing the light and heavy chains in a single host cells are known, see, for example, U.S. Patent No. 4,816,397, European patent application publication No. 88,994, PCT published patent application WO 93/19172, U.S. Patent No. 4,816,567, U.S. Patent No. 4,975,369, U.S. Patent No.

5,202,238, PCT published patent application WO 86/01533, PCT published patent application WO 94/02602, and European published patent application No. 273,889.

Vector constructs

The vectors of the invention are recombinant DNA vectors including, but not limited to, plasmids, phages, phagemids, cosmids, viruses, retroviruses, and the like, which insert a nucleotide sequence into a cell.

Methods for introducing an exogenous nucleotide sequence of interest into a cell, including into antibody-producing cells, are known in the art. These methods typically include use of a DNA vector to introduce the nucleotide sequence into the genome or a cell or cells, and then growing the cells to generate a suitable population. Nucleotide sequences may also be introduced directly into a cell by methods known in the art.

In a preferred embodiment, nucleotide sequences are introduced into mammalian cells according to the electroporation transfer procedure described by Neumann et. al. (1982) EMBO J. 1:841, herein specifically incorporated by reference. In another preferred embodiment, nucleotide sequences are introduced into mammalian cells according to the liposome-mediated transfer procedure described by Felgner et. al. (1987) PNAS 84:7413, herein specifically incorporated by reference. Lipofection is particularly preferred when the host cells are myeloma cells. Transfection of mammalian cell lines may be accomplished by any of a number of methods known to those skilled in the art, including but not limited to microinjection, $CaPO_4$ precipitation, RBC ghost fusion, protoplast fusion, and the like.

DNA sequences

The nucleotide sequence encoding a component of the desired multi-component protein may be obtained as a cDNA or as a genomic DNA sequence by methods known in the art. For example, messenger RNA coding for a desired component may be isolated from a suitable source employing standard techniques of RNA isolation, and the use of oligo-dT cellulose chromatography to segregate the poly-A mRNA. When the product multi-component protein is an antibody, suitable sources of desired nucleotide sequences may be isolated from mature B cells or a hybridoma culture.

In addition to the nucleotide sequence encoding the desired component of the product multi-component protein, vector constructs can include additional components to facilitate replication in prokaryotic and/or eukaryotic cells, integration of the construct into

a eukaryotic chromosome, and markers to aid in selection of and/or screening for cells containing the construct (e.g., the detectable markers and drug resistance genes discussed above for the targeting construct). For eukaryotic expression, the construct should preferably additionally contain a polyadenylation sequence positioned 3' of the gene to be expressed. The polyadenylation signal sequence may be selected from any of a variety of polyadenylation signal sequences known in the art. Preferably, the polyadenylation signal sequence is the SV40 early polyadenylation signal sequence.

Transformation of host cells

Antibodies have been expressed in a variety of host cells, including bacterial, yeast, and insect cells. For the production of large, multimeric proteins, mammalian cell expression systems generally provide the highest level of secreted product (Bebbington (1991) Methods: A Companion to Methods Enzymol. 2:136-145). Myeloma cells have been used as fusion partners for splenic cells to generate hybridomas cells expressing antibodies. Transformed myeloma cells may be used as fusible host cells in the method of the invention.

Host cells

Nonlymphoid cells lines have been investigated for use in producing antibodies (Cattaneo & Neuberger (1987) EMBO J. 6:2753-2758; Deans et al. (1984) Proc. Natl. Acad. Sci. 81:1292-1296; Weidle et al. (1987) Gene 51:21-29). The ability of nonlymphoid cell lines to assemble and secrete fully functional antibodies may be exploited for antibody production. For example, Chinese hamster ovary (CHO) cells, Chinese Hamster lung (V79) cells (Elkind et al. (1960) Radiat. Res. 13:556), and COS cells have well-characterized efficient expression systems and have been used for both long-term and transient expression of a variety of proteins (Bebbington (1991) supra; Rauschenbach et. al. (1995) Eur. J. Pharm. 293:183-90). A method for achieving a high level of expression of DNA sequences encoding a chimeric antibody in transformed NS0 myeloma cells has been described (Bebbington et al. (1992) Bio/Technology 10:169-175).

Any mammalian cell line capable of expressing the desired multimeric protein and amenable to fusion is suitable for use in the present invention. For example, where the desired protein is an antibody, the cell line is any mammalian cell capable of expressing a functional antibody. A preferred host cell is a mammalian myeloma cell; most preferably, an non-secreting (NS) myeloma cell (e.g., a non-secreting (NSO) myeloma). Other

myeloma cells include mouse derived P3/X63-Ag8.653, P3/NS1/1-Ag4-1(NS-1), P3/X63Ag8.U1 (P3U1), SP2/O-Ag14 (Sp2/O, Sp2), PAI, F0, and BW5147; rat derived 210RCY3-Ag.2.3; and human derived U-266AR1, GM1500-6TG-A1-2, UC729, CEM-AGR, DIR11, and CEM-T15.

Selection of transformed cells

Detection of transfectants with properly integrated vector sequences can be accomplished in a number of ways, depending on the nature of the integrated sequences. If the transferred nucleotide sequence includes a selectable marker, the initial screening of the transfected cells is to select those which express the marker. Any of a variety of selectable markers known in the art may be included in the construct, including dihydrofolate reductase (DHFR), guanosine phosphoryl transferase gene (*gpt*), neomycin resistance gene (*Neo*), hygromycin resistance gene (*Hyg*) and hypoxanthine phosphoribosyl transferase (HPRT). For example, when using a drug resistance gene, those transfectants that grow in the selection media containing the drug (which is lethal to cells that do not contain the drug resistance gene) can be identified in the initial screening. It will be appreciated that a variety of other positive, as well as negative (i.e., HSV-TK, cytosine deaminase, and the like), selectable markers that are well known in the art can be utilized in accordance with the present invention for selection of specific cells and transfection or other events. As well, a variety of other marker genes (i.e, the LacZ reporter gene and the like) can be utilized in similar manners.

After a period of time sufficient to allow selection to occur (in most cases, about 2 weeks) the surviving cells are then subjected to a second screening to identify those transfectants which express the desired peptide component of interest. This may be accomplished by, for instance, an immunoassay using antibodies specific for the particular immunoglobulin class.

The protocol for the second screening depends upon the nature of the inserted sequences. For example, where the cell is transformed with a sequence which does not result in a secreted product, selection for the presence of the foreign DNA can be detected by Southern blot using a portion of the exogenous sequence as a probe, or by polymerase chain reaction (PCR) using sequences derived from the exogenous sequence as amplifiers. The cells having an appropriately integrated sequence can also be identified by detecting expression of a functional product, e.g., immuno-detection of the product. Alternatively,

the expression product can be detected using a bioassay to test for a particular effector function conferred by the exogenous sequence.

Where the first host cell is transfected with DNA encoding heavy chain, the expression of the heavy chain can be tested using any conventional immunological screening method known in the art, for example, ELISA conducted with cell lysate samples (see, for example, Colcher et al. Protein Engineering 1987 1:499-505). The cell can be further selected for additional desirable characteristics such as heavy chain production rate or level, ability of the expressed heavy chain to combine with light chain to provide an antibody of a desired antigen binding affinity, and other characteristics desirable for heavy chain production and heavy chain function in an antibody.

Nonlymphoid cells expressing a desired protein may be transfected in a number of ways known to the art. One example of the method of the invention is described in Example 1 below. A first CHO cell may be transfected with a vector comprising a DNA sequence encoding a desired light chain and a second CHO cell transfected with a vector comprising a DNA sequence encoding a desired heavy chain. Transfected cells are selected and fused. Fused cells are selected for expression of an antibody having the desired light chain Ig and heavy chain Ig.

Similarly, a nonlymphoid cell such as V79 may be used as the host cell in the method outlined above. This example of the method of invention is described in Example 2 below.

In one embodiment, a cell expressing an Ig heavy chain gene also expresses an irrelevant Ig light chain gene. In some instances, co-expression of a light chain may be required for secretion and expression of the Ig heavy chain. Failure of a cell to secrete the heavy chain peptide may make detection of transfectants more difficult since it necessitates assaying the cells themselves (e.g., by Northern blot analysis or immuno-detection), as opposed to conveniently screening the cell supernatant by ELISA.

In a specific embodiment of the invention, this problem is avoided by transfecting a first host cell expressing an irrelevant light chain with a plasmid bearing the desired heavy chain (Fig. 3). The gene encoding the irrelevant light chain may either be integrated into a chromosome or be present in an episomal vector, such as bovine papilloma virus (BPV) or other episomal vector known in the art. After selection for transformants, expression of

the heavy chain is easily confirmed by an ELISA assay of the cell lysates for secreted antibody.

Cells expressing the desired heavy chain are then fused with a second cell that has been transfected with the desired light chain under appropriate fuseogenic conditions according to methods well known in the art (see, e.g., Harlow & Lane, supra). Any combination of cells capable of expressing a desired heavy chain or desired light chain and that can be fused to produce a hybrid cell expressing both heavy and light chains can be used. Thus, the first cell (e.g., expressing the desired heavy chain) can be of the same or different type as the second cell (e.g, expressing the desired light chain), e.g., the first cell can be a myeloma cell and the second cell can be a non-lymphoid cell. The fusion product cells which are candidates for manufacturing lines will express the desired heavy chain and light chain, but will have lost the irrelevant light chain. During the fusion process, random chromosomes are normally lost. Thus, it is expected that cells lacking the irrelevant Ig light chain will be generated during the fusion process. These hybrid cells can easily be identified by ELISA assay of the supernatants for the presence of the desired chains and absence of the irrelevant chain.

Thus, in one embodiment, the desired light chain of the final antibody product is the κ light chain. In such cases, a Igλ expressing myeloma cell is transfected with the desired IgH gene. After transfection with a plasmid carrying the desired heavy chain and selection, cells expressing the heavy chain are examined directly for expression of the desired heavy chain, e.g., ELISA assay of the supernatants with antibody specific to the heavy chain. The second cell, e.g., a non-secreting myeloma cell, is transfected with the κ light chain, and transfectants detected through e.g., Northern blot analysis or immuno-detection with an antibody specific to the κ light chain. The cells expressing the light chain can be further selected for desirable characteristics associated with production of a functional light chain, such as light chain production rate or level, ability of the expressed light chain to combine with heavy chain to provide an antibody of a desired antigen binding affinity, and other characteristics desirable for light chain production and heavy chain function in an antibody. The cells are then fused, and the hybrid cell expressing the desired IgH/IgK antibody is selected for the presence of the κ light chain and desired heavy chain (e.g., C_γ) and the absence of λ light chain, e.g., by ELISA assay of the culture medium.

When the desired product antibody contains a λ light chain, the first cell transfected with DNA encoding the desired heavy chain will express a κ light chain, and final selection of hybrid cells expressing the desired antibody will select for the presence of the λ light chain and the absence of the κ light chain.

In an alternative embodiment of the invention, selection of fused or hybrid cells can be initially determined through the utilization of distinct marker genes in each of the "parental" cells or cell lines. Such technique is shown in Figure 4. There, a parental CHO cell line, that is DHFR, is transfected with a vector (pManu Kappa) that contains the DHFR resistance gene and the hygromycin resistance gene (HYGRO). Another parental CHO cell line, that is DHFR, is transfected with a vector (pManu Gamma) that contains the DHFR resistance gene and the neomycin resistance gene. Each cell line, following transfection, contains distinct selectable markers (i.e., hygromycin resistance in the first and neomycin/G418 resistance in the second). Thus, upon fusion, resulting "daughter" cells in which fusion has been successful will be resistant to both hygromycin and G418. The screening technique of the invention is advantageous in that it mitigates the need to determine expression of immunoglobulin molecules in order to determine if a fusion has been successfully performed.

Under certain fusion conditions, cells and cell lines can become spontaneously resistant to G418, and, possibly, other selectable markers. Thus, in certain embodiments of the invention, it is preferable to utilize selectable markers to which cells and cell lines are less likely to spontaneously generate resistance. An example of one such marker is the hypoxanthine phosphoribosyl transferase gene (HPRT) which confers resistance to hypoxanthine aminopterin. Another marker that can be used in tandem with HPRT resistance is the LacZ gene. The LacZ gene is not a selectable marker; but, rather, acts as a marker gene which, when expressed by a cell, stains blue in the presence of β-galactosidase.

Thus, through following a similar scheme as described in connection with Figure 4, a first parental cell line, which is HPRT deficient (such as the P3X, NSO, and NSO-bcl2 myeloma cell lines), is transfected with an antibody gene cassette. The cassette includes, for example, appropriate antibody genes, a gene amplification system, and an HPRT selectable marker. Transfected cells can be selected through HPRT selection and cells producing high levels of antibodies can be picked. A second parental cell line, which is

also preferably HPRT deficient, is transfected with an antibody gene cassette. The cassette includes, for example, appropriate antibody genes, a gene amplification system, and the LacZ gene. Transfected cells can be selected through staining with β-gal. As will be appreciated, either the first or second parental cell line can include the light chain genes or the heavy chain genes and the other of the first or second parental cell line will contain the other of the light or heavy chain genes. As will also be appreciated, other selectable markers can be included in the cassettes utilized to transfect the cells. Upon fusion of the first and second parental cell lines, successful fusion can be determined through HPRT selection and β-gal staining of daughter cells. Daughter cells can be further selected based upon expression levels of immunoglobulin molecules.

Specific embodiments of this technique is illustrated in Figure 5 in several exemplary schemes. In the Figure, a first parental cell line, exemplified by the myeloma cell line, NSO, which is HPRT deficient, is transfected with a light chain cassette containing a gene amplification system (AM), an antibody light chain gene system ($V_KJ_KC_K$), and an HPRT selectable marker (HPRT) (Step 1). A second parental cell line, exemplified by any one of J558L, Ag.1, or NSO, are transfected with a heavy chain cassette containing a gene amplification system (AM), an antibody heavy chain gene system ($V_HD_HJ_Hh\gamma$), and the LacZ gene (Step 2). The transfection of J558L cell line is indicated as Step 2a, the transfection of the Ag.1 cell line is indicated as Step 2b, and the transfection of the NSO cell line is indicated as Step 2c. With respect of each Step 1 and Steps 2a-2c, the success of the transfection can be determined through the use of the selectable marker HPRT in Step 1 and through β-gal staining in connection with each of Steps 2a-2c. Additionally cells can be picked for expression of light chain (Step 1) or heavy chain (Step 2a-2c).

Following isolation and generation of parental cell lines incorporating the antibody gene cassettes, fusion between a parental cell line including heavy chain genes and a parental cell line including light chain genes is conducted. Utilizing techniques described herein, the parental cell line resulting from Step 1 is fused with a parental cell line resulting from Steps 2a-2c. This is indicated in the Figure as fusion 1-2a, fusion 1-2b, and fusion 1-2c, which results in fused cells 1-2a, 1-2b, and 1-2c, respectively. Such fused cells can be readily identified through dual marker selection, that is, HPRT selection and

β-gal staining. Cells which have been successfully fused, will be HPRT resistant and will stain positive with β-gal.

As will be appreciated, the parental cell lines utilized in fusions 1-2a and 1-2b additionally contain mouse mλ and rat γK genes. Thus, daughter cells from fusions 1-2a and 1-2b are preferably selected to ensure that they are mλ and γK. Loss of mouse mλ genes and rat γK genes will generally occur naturally through recombination events during the fusion process.

EXAMPLES

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use various constructs and perform the various methods of the present invention and are not intended to limit the scope of what the inventors regard as their invention. Unless indicated otherwise, parts are parts by weight, temperature is in degrees centigrade, and pressure is at or near atmospheric pressure. Efforts have been made to ensure accuracy with respect to numbers used, (e.g., length of DNA sequences, molecular weights, amounts, particular components, etc.) but some deviations should be accounted for.

Example 1. Generation of Hybrid Cells Containing Light and Heavy Ig Chains.

The human heavy chain Ig construct (IgH gamma) was ligated into the pManugamma#6 vector (Fig. 4; Cell Genesys, Inc., Foster City, CA) containing DHFR and neo marker genes. The human kappa light chain Ig construct was ligated into the pManukappa#14 (Fig. 4; Cell Genesys, Inc.) which contains DHFR and hygromycin resistance marker genes. The Ig constructs were derived from a hybridoma which secretes an IL-8 antibody.

Overview of the Cell Fusion Method

In general, the experiment proceeds as follows: A first cell is transfected with the pManukappa vector comprising the human kappa light chain transgene, and MTX and hygromycin selection marker genes. A second cell is transfected with the pManugamma vector comprising a human γ_4 heavy chain transgene and Neo and MTX selectable marker genes. After the appropriate selection and amplification, the selected first and second cells are fused to form the hybrid cell of the invention expressing a human antibody.

Cell Transfection

Chinese hamster ovary (CHO) cells are transfected by electroporation as follows: DHFR-deficient CHO cells in exponential growth are fed with growth medium 4 hours prior to electroporation [growth medium: DMEM/Ham's F12 (50:50 mixture; JRH BioSciences, Woodland, CA), 10% FBS, 2 mM glutamine, non-essential amino acids (NEAA) plus glycine, hypoxanthine and thymidine (GHT)]. Cells are collected, washed in PBS, and resuspended in PBS to a concentration of 5 x 10^6 cells per 0.8 ml. The cell suspension is aliquoted into 0.4 cm electroporation cuvettes (0.8 ml per cuvette) and 5-20 μg linearized DNA added. The suspension is mixed and left on ice for 10 min. Each cuvette is electroporated at 260 V and 960 μF. Each cuvette is place on ice for 10 min, the cells resuspended in 20 ml growth medium, then plated onto 2 10 cm cell culture plates. After 48 hrs, cells from each culture plate are replated in 10 culture plates in the presence of selective media [DMEM, 4.5 g/l glucose (JRH Biosciences), 10% dialyzed FBS (Life Technologies, Bethesda, MD), 5 mM glutamine, NEAA, 0.6 mg/ml G418].

Selection of Transfectants

Cells transfected with the kappa light chain transgene were selected in the presence of methotrexate (MTX) and hygromycin. Cells were plated 48 hr post-electroporation into 10 plates in DHFR selective media [DMEM, 4.5 g/l glucose (JRH Biosciences), 10% dialyzed FBS (Life Technologies, Bethesda, MD), 5 mM glutamine, NEAA, supplemented with hygromycin (Calbiochem, San Diego, CA) at concentrations ranging from 250-750 μg/ml]. Recombinant protein expression can be increased by DHFR-mediated amplification of the transfected gene. Methods for selecting cell lines bearing gene amplifications are known in the art, e.g., for example, as described in Ausubel et al. (1989) Current Protocols in Molecular Biology, John Wiley & Sons, New York; such methods generally involve extended culture in medium containing gradually increasing levels of methotrexate.

Heavy chain transfectant CHO cells are selected in the presence of MTX and neomycin following the above described procedures.

Generation of a Hybrid Cell Expressing an Antibody

Prior to fusion, PEG/DMSO fusion solution (50% PEG, 10% DMSO in PBS)(Sigma) is placed in a 37°C incubator overnight, and 500-1000 ml incomplete Ham's F12 solution (without FCS) is filtered. At fusion, warm fusion medium and incomplete

DMEM/Hams' F12 are placed in a 37°C water bath. A water-filled beaker and a 15 ml conical tube filled with incomplete DMEM/Ham's F12 are also placed in the water bath. Harvested transfected CHO cells are washed once with incomplete DMEM/Ham's F12, pelleted at 1200 rpm, resuspended in incomplete DMEM/Ham's F12, and counted. The kappa light chain and the γ_4 transfected cells are mixed in a 1:1 ratio, and centrifuged at 800 x g (2060 rpm). The following fusion steps are followed: (1) add 1 ml PEG/DMSO fusion solution to cells over 1 min period; (2) stir cells gently for 1 min; (3) add 2 ml incomplete DMEM/Ham's F12 over a 2 min period with slow stirring; and (4) add 8 ml of incomplete DMEM/Ham's F12 over a 3 min period with slow stirring. The cells are then centrifuged at room temperature at 400 x g for 5 min (1460 rpm). Selection medium [complete DMEM/Ham's F12 + 10% FCS + 250-750 μg/ml hygromycin + 0.6 mg/ml G418] is added to the cell pellet. 10 ml of selection medium are added to the cell pellet; cells are gently stirred to resuspend.

The cells are plated onto 10 cm dishes as dilutions of 1:10, 1:20, and 1:40 in selection medium. The plates are refed with fresh medium every 3 days until clones appear. Clones are picked and transferred to a 96-well plate in selection medium. As will be appreciated, growth of cells to reach confluence, which demonstrates survival of cells through selection with hygromycin and G418, is indicative that the cells contain both the heavy and light chain Ig genes, since hygromycin resistance was contributed by the light chain gene containing parental cells and neomycin resistance was contributed by the heavy chain gene containing parental cells. As such, dual marker selection provides an expedient method to initially determine whether a fusion has been successfully accomplished. Following such an initial screen, supernatant can be assayed for expression of the desired antibody as described below. When the wells are confluent, the supernatant may then be assayed for expression of the desired antibody as described below.

Selection for Desired Hybrid Cell

Expression of the desired antibody may be assayed by immunological procedures, such as Western blot or immunoprecipitation analysis of hybrid cell extracts, or by immunofluorescence of intact cells (using, e.g., the methods described in Ausubel et al. (1989) supra). The desired antibody can be detected using antibody specific for each component of the desired antibody, e.g., antibodies specific to the kappa light chain and γ_4 heavy chain.

Confirmation of Desired Characteristics of Antibody Produced by Hybrid Cell

After the hybrid cell is produced and antibody production in the cell is confirmed, the hybrid cell is grown under conditions to allow expression of the antibody and secretion of the antibody into the cell culture supernatant. For example, the cells can be grown in roller bottles in selective growth medium (DMEM/Ham's F12 (50:50 mixture), 10% FBS, 2 mM glutamine, non-essential amino acids plus glycine, hypoxanthine and thymidine, plus hygromycin and G418 to provide continued selection for the heavy and light chain constructs in the hybrid cell) for several hours prior to assay. Cell culture supernatant is collected and the antibodies are tested for various desired characteristics, e.g., antigen binding affinity (e.g., preferably antigen binding affinity that is similar to that of the original antibody from which the recombinant antibody is derived) using immunological assays well known in the art (e.g., ELISA, or competition binding assays).

Example 2. Generation of Hybrid Cells Containing Light and Heavy Ig Chains.

According to the known conventional genetic engineering methods, an IgK expression vector (pLS413) and an IgH expression vector (pLS421) were constructed as shown in Fig. 6. Each of the constructs was introduced into the host cells to prepare IgK-producing cells and IgH-producing cells. The light chain gene and the heavy chain gene were cloned from a hybridoma, D39.2, which produces and anti- human IL-8 antibody. Hybridoma D39.2 was prepared from a mouse engineered to produce human antibody ((1994) Nature Genetics, 7:13-21).

In the IgK expression vector, the light chain gene encoding the human variable and human constant regions was inserted. For class-switching, the gene encoding the constant region of the heavy chain gene was replaced with the gene encoding the human $C\gamma_4$ constant region. Then, in the IgH expression vector, the heavy chain gene encoding the human variable and human constant $C\gamma_4$ regions was inserted. The IgK and IgH expression vectors have gpt gene and neomycin resistance gene as selectable markers, respectively. The vectors were digested with Sal I to linearize before electroporation.

IgK producing cell

6-Thioguanine resistant V79 cells (1×10^7) were transfected with 100 μg of the linearized IgK expression vector by electroporation with pulse strength of 300V/0.4 cm and 960 μF. The cells were cultivated with Minimum Essential Medium (MEM) containing 10 % Fetal Bovine Serum (FBS) and HAT (100 μM hypoxanthine, 0.4 μM

aminopterin, 16 μM thymidine). Fifteen days after the transfection, more than 1000 clones grew. Twenty clones were selected. After 10 to 20 days cultivation, IgK concentration in the medium was measured by ELISA as shown in Table 1. All clones produced IgK except K-17, which did not grow.

Table 1: IgK producing cells

Clone	Concentration (ng/ml)
K-1	1,250
K-2	750
K-3	1,250
K-4	1,250
K-5	200
K-6	2,000
K-7	1,250
K-8	1,250
K-9	500
K-10	250
K-11	200
K-12	1,200
K-13	200
K-14	1,500
K-15	500
K-16	700
K-18	700
K-19	1,000
K-20	50

IgH producing cell

6-thioguanine resistant V79 cells ($1x10^7$) were transfected with 100 μg of the linearized IgH expression vector by electroporation with pulse strength of 300V/0.4 cm and 960 μF. The cells were cultivated with MEM containing 10% FBS and G418 (400 μg/ml). Fifteen days after the transfection, more than 400 clones grew. Eleven clones were selected. Seven days after the initial culture, a lysate of 1×10^5 cells of each clone was prepared. As the IgH alone is not secreted into the medium, IgH in the lysate was measured by ELISA. Seven out of eleven clones produced IgH as shown in Table 2.

Table 2: IgH producing cells

Clone	IgH (ng/ $1x10^5$ cells)
H-1	25
H-2	25
H-3	0
H-4	0
H-5	25
H-6	50
H-7	0
H-8	20
H-9	0
H-10	50
H-11	12

Fusion process

The IgK producing cell, clone K-15 (2×10^5) and the IgH producing cell (2×10^5) were mixed and cultured for one night in a dish (10 cm diameter). After removing the medium, the cells were treated with 4 ml 50% PEG 1500 solution for one minute. The cells were washed with fresh MEM five times to remove PEG completely. The cells were removed by trypsin treatment and were suspended. The cells were transferred onto four plates (10 cm diameter) and were cultivated with MEM containing 10% FBS, HAT (100 μM hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine), and G418 (100 μg/ml) for 15 days. Eighteen clones were selected. After ten to twenty days cultivation, IgG concentration in the medium was measured by ELISA. As shown in Table 3, each fused cell makes IgG except the F-5 clone, which did not grow.

Table 3: Antibody-producing cells (following fusion)

Clone	Concentration (ng/ml)
F-1	98
F-2	347
F-3	132
F-4	85
F-6	1,133
F-7	124
F-8	292
F-9	310
F-10	97
F-11	264
F-12	290
F-13	408
F-14	193
F-15	1,258
F-16	654
F-17	324
F-18	203

Antigen specificity of the IgGs were examined by ELISA. The plate used was coated with human IL-8, and anti-human IgK antibody conjugated with peroxidase was used for detection in the ELISA. As shown in Figure 7, all IgGs were human IL-8 specific with similar affinity of the original antibody, D39.2.

Example 3.

The following is an example of another fusion process that has been utilized in accordance with the invention:

Preparation of cells

Prior to fusion, parental cell lines for use in the fusion are grown up and maintained in medium containing DMEM high, 10% FBS, 1% non-essential amino acids, 1% pen-strep, and 1% L-glutamine.

On the day prior to fusion, each of the parental cell lines are prepared and split to provide a cell density of approximately 10^5 cells/ml. On the day of the fusion, cells are counted and the fusion is commenced when, and assuming, that cell count for each of the parental cell lines are within the range of about 1.5-2.5 x 10^5 cells/ml. Sufficient quantities of each of the parental cell lines to make up 5 x 10^6 cells each are withdrawn

from the cultures and added to a 50ml centrifugation tube and the cells are pelleted at 1200 rpm for approximately 5 minutes. Concurrently with the preparation of the cells, incomplete DMEM, PEG, and double selection media are prewarmed in a 37°C incubator bath. Following pelleting, cells are resuspended in 20 ml incomplete DMEM and pelleted again. Thereafter, the cells are resuspended in 5 ml incomplete DMEM and the two parental cell lines are pooled in a single tube and pelleted again to form a co-pellet containing both of the parental cell lines. The co-pellet is resuspended in 10 ml incomplete DMEM and again pelleted. All of the supernatant is then removed from the co-pellet and the cells are ready for fusion.

Fusion

Following removal of all of the supernatant, 1 ml PEG-1500 is added over the course of 1 minute to the co-pellet while stirring. After addition of the PEG is completed, either gentle stirring with a pipet is continued for 1 minute or the suspended co-pellet can be allowed to stand for 1 minute. Thereafter, 10 ml of incomplete DMEM is added to the co-pellet over the course of 5 minutes with slow stirring. The mixture is then centrifuged at about 1200 rpm for 5 minutes and following centrifugation, the supernatant is aspirated off, and 10 ml of complete double selection medium is added and gently stirred into the cells. The cells are then plated at 100 μl/well into 10 96-well microtiter plates and placed into an incubator (37^0 C with 10% CO_2) where they are not disturbed for 1 week. After the passage of a week, plates are fed by adding 100 μl of complete double selection medium to each well.

Clones surviving selection are isolated and productivity assays conducted in accordance with Example 4. Clones may be further subjected to limited dilution cloning using standard techniques.

Double selection medium is prepared depending upon the marker gene utilized in connection with the parental cell lines. In the majority of our experiments, the selectable xx markers conferring puromycin, hygromycin, or hypoxanthine aminopterine (HAT) resistance are utilized. Concentrations required to obtain complete cell killing of NS/0 cells were determined through use of kill curves and resulted in our use of 6 micrograms/ml of puromycin and 350 micrograms/ml of hygromycin. In connection with HPRT resistance, we used HAT media supplement from Sigma using standard conditions.

Example 4.

In this Example, a productivity assay is provided for the analysis of Ig expression by daughter cells obtained through the fusion process. In the assay, cells are counted and 200,000 are selected and washed with complete DMEM media (containing 10% fetal calf serum, 1% glutamine, 1% nonessential amino acids, and 1% pen-strep). The cells are pelleted (at about 1200 rpm for about 5 minutes) and the supernatant is removed. The cells are resuspended in 2 ml medium and plated in a 6-well plate. The cells are then grown at 37^0 C with 10% CO_2 in an incubator for 4 days. Thereafter cells are resuspended and counted using a hemacytometer. Cells are pelleted and the supernatant is retained for ELISA.

The ELISA is conducted using a human κ capture, followed by detection with a polyclonal human anti-IgG. Standards for the ELISA are isotype specific. The ELISA provides a quantitative measurement of the amount of secreted antibody. Through starting with a known number of cells and obtaining the number of cells after four days of growth, we can also estimate the antibody production per cell.

Example 5

In this example, the generation of parental cell lines containing either a human heavy chain construct or a human κ light chain construct is described.

Construct Generation

In connection with generation of either parental cell line, the construct shown schematically in Figure 8 was used. For preparation of the human Ig cassette, a human anti-IL-8 IgG_2 monoclonal antibody producing hybridoma (designated D1.1) was utilized for source DNA. The D1.1 hybridoma was derived from a mouse engineered to produce human antibodies as described in Mendez et al. *Nature Genetics* 15:146-156 (1997) and U.S. Patent Application Serial No. 08/759,620, filed December 3, 1996 (the disclosures of which are incorporated by reference in their entirety) that was immunized with human IL-8.

Each of the heavy chain variable region and the entire κ light chain was cloned from the D1.1 hybridoma using RT-PCR and cDNA obtained. In the heavy chain construct, separately, genomic DNA encoding a human gamma-4 constant region was isolated and cloned and ligated to the cDNA encoding the heavy chain variable region.

Each of the heavy chain construct and the light chain construct were ligated into the vector shown schematically in Figure 8 to form constructs H/pEE12.1 (heavy chain) and K/pEE12.1 (light chain). Such constructs were then fitted with appropriate selectable marker cassettes. For example, puromycin resistance was provided to the heavy chain construct and hygromycin resistance was provided to the light chain construct to form the vectors H/Pur/pEE12.1 and K/Hyg/pEE12.1, respectively, and as shown in the restriction maps provided in Figures 9 and 10, respectively.

Transfection

We prepared an NS/0 and NS/0-bcl-2 myeloma cell line containing the human heavy chain construct (H/Pur/pEE12.1) and NS/0 and NS/0-bcl-2 cell lines containing the human κ light chain construct (H/Hyg/pEE12.1) through standard transfection techniques.

In connection with the heavy chain construct, transfection was accomplished through linearizing the H/Pur/pEE12.1 construct at the PacI restriction site and electroporating 20 μg of the same into 5 x 10^6 cells (either NS/0 or NS/0-bcl-2 cells). Electroporation was accomplished at 300 V, 960 μF. After 10 minutes at room temperature, cells were plated in 10 ml DMEM complete media in P100 plates. In connection with the light chain construct, the same techniques were used, however, the DNA was linearized using the SalI restriction ste.

After 24 hours recovery, viability was assayed with trypan blue. Cells were harvested, pelleted, resuspended in selection medium, and plated at 5,000-10,000 viable cells per well in 96-well plates, either in medium containing puromycin (heavy chain) or hygromycin (light chain). Clones surviving selection were isolated and expanded. Light chain and heavy chain production from cells surviving selection were measured using ELISA and normalized to total protein assayed by Bradford on cell lysates. Heavy chain the ratio of ELISA OD to total protein OD in NS/0 cell lines ranged from about 3.0 to 7.5 total protein (NS/0-bcl-2 cell lines containing heavy chains were not assayed). Light chain the ratio of ELISA OD to total protein OD in NS/0 cell lines ranged from about 0.5 to 5.99 total protein and NS/0-bcl-2 cell lines ranged from about 0.3 to 6.28. Based upon the above assay, the highest producing single heavy chain containing NS/0 cell line (7.5), the highest producing light chain containing NS/0 cell line (5.99), and the highest producing NS/0-bcl-2 cell line (6.28) were selected for use in fusions.

Example 6

In this example, results obtained through use of the fusion process described in connection with Example 3 are provided (Table 4)experiment, a human heavy chain Ig containing NS/0 parental cell line was fused with a human κ light chain containing NS/0 parental cell line. Thus, in the experiment, an NS/0 cell line containing the heavy chain VDJ gamma-4 cassette and a puromycin resistance cassette was fused with an NS/0 cell line containing the human kappa light chain cassette and the hygromycin resistance cassette. Fusion and selection were accomplished using the procedure described in Example 3 and productivity determined in accordance with Example 4.

Table 4

Clone (1^{st} subcloning)	μg/ml	pg/cell	Clone (2^{nd} subcloning)	μg/ml	pg/cell
54.2	17.5	14.8	54.2.1	25.3	20.7
			54.2.2	28.4	29.6
			54.2.3	43.6	43.6
			54.2.4	16.6	24.8
			54.2.5	5.0	11.4
			54.2.6	11.1	15.0
88.5	8.3	8.1	88.5.1	9.7	9.9
			88.5.2	10.0	17.8
			88.5.3	10.1	15.5
			88.5.4	3.5	3.6
			88.5.5	16.3	21.4
			88.5.6	3.7	3.2
91.2	7.3	5.1	91.2.1	4.1	4.8
			91.2.3	3.5	4.4
			91.2.4	22.5	28.1
			91.2.5	12.1	16.8
			91.2.6	6.9	11.9
96.5	7.5	8.3	96.5.1	7.9	10.6
			96.5.2	15.7	18.9
			96.5.4	6.1	6.1
			96.5.5	7.6	7.6
			96.5.6	14.2	18.9

Example 7

In this example, results obtained through use of the fusion process described in connection with Example 3 are provided (Table 5). In the experiment, a human heavy chain Ig containing NS/0 parental cell line was fused with a human κ light chain

containing NS/0-bcl-2 parental cell line. Thus, in the experiment, an NS/0 cell line containing the heavy chain VDJ gamma-4 cassette and a puromycin resistance cassette was fused with an NS/0-bcl-2 cell line containing the human kappa light chain cassette and the hygromycin resistance cassette. Fusion and selection were accomplished using the procedure described in Example 3 and productivity determined in accordance with Example 4.

Table 5.

Sample ID	hIgG/hκ Concentration (μg/ml)
1 2.2.1	4.75
2 2.2.2	3.18
3 2.2.3	3.57
4 2.2.4	3.19
5 2.2.5	0.559
6 2.2.6	5.05
7 27.6.3	9.87
8 27.6.4	11.7
9 29.1.3	14.2
10 29.1.4	4.61
11 29.1.6	12.9
12 23B3	0.952

Example 8

In this example, results obtained through use of the fusion process described in connection with Example 3 are provided (Table 6). In the experiment, a human heavy chain Ig containing NS/0 parental cell line was fused with either a human κ light chain containing NS/0-bcl-2 parental cell line (Clone ID's 17, 19, and 20) or a human κ light chain containing NS/0 parental cell line (Clone ID's 29, 37, and 39). Thus, in the experiment, an NS/0 cell line containing the heavy chain VDJ gamma-4 cassette and a puromycin resistance cassette was fused with an NS/0 or NS/0-bcl-2 cell line containing the human kappa light chain cassette and the HPRT (HAT resistance) cassette. Fusion and selection were accomplished using the procedure described in Example 3 and productivity determined in accordance with Example 4.

Table 6

Clone ID	mg/ml Ig	total mg Ig	# cells	Total #	pg/cell
17.1	9.76	19.52	1,020,000	2040000	9.57
17.3	4.68	9.36	700,000	1400000	6.69
17.4	6.40	12.8	610,000	1220000	10.49
19.1	15.90	31.8	890,000	1780000	17.87
19.2	16.80	33.6	900,000	1800000	18.67
19.3	12.20	24.4	790,000	1580000	15.44
19.4	16.20	32.4	840,000	1680000	19.29
20.1	13.00	26	910,000	1820000	14.29
20.2	6.12	12.24	840,000	1680000	7.29
20.3	7.35	14.7	1,120,000	2240000	6.56
20.4	17.00	34	1,000,000	2000000	17.00
29.1	0.19	0.372	960,000	1920000	0.19
29.2	0.05	0.1	930,000	1860000	0.05
29.3	6.02	12.04	900,000	1800000	6.69
29.4	0.33	0.652	1,400,000	2800000	0.23
37.1	0.05	0.1	910,000	1820000	0.05
37.2	2.30	4.6	1,430,000	2860000	1.61
37.3	0.01	0.024	1,000,000	2000000	0.01
37.4	5.12	10.24	1,400,000	2800000	3.66
39.1	0.29	0.572	1,400,000	2800000	0.20
39.2	0.66	1.31	1,160,000	2320000	0.56
39.3	0.29	0.572	1,000,000	2000000	0.29
39.4	0.31	0.624	1,000,000	2000000	0.31

The instant inventions shown and described herein are what is considered to be the most practical and the preferred embodiments. It is recognized, however, that departures may be made therefrom which are within the scope of the invention, and that obvious modifications will occur to one skilled in the art upon reading this disclosure.

CLAIMS

What is claimed is:

1. A method for producing a multi-component protein, said method comprising:

(a) introducing a first nucleotide sequence into a first cell, wherein the first nucleotide sequence encodes a first component of the multi-component protein;

(b) introducing a second nucleotide sequence into a second cell, wherein the second nucleotide sequence encodes a second component of the multi-component protein;

(c) optionally, repeating step (b) for each remaining component of the multi-component protein; and

(d) fusing cells produced from steps (a)-(c) to form a hybrid cell, wherein the hybrid cell expresses the multi-component protein.

2. The method of claim 1, further comprising:

(e) culturing the hybrid cells so as to express the multi-component protein; and

(f) recovering the multi-component protein from the hybrid cell culture.

3. The method of claim 1, wherein said first cells and said second cells are selected from the group consisting of a mammalian cell, a myeloma cell, and a non-lymphoid cell.

4. The multi-component protein of claim 1, wherein said protein is an antibody.

5. A method for producing an antibody, said method comprising:

(a) introducing a nucleotide sequence encoding a desired heavy chain into a first cell;

(b) introducing a nucleotide sequence encoding a desired light chain into a second cell; and

(c) fusing the first and second cells to form a hybrid cell, wherein the hybrid cell expresses the antibody.

6. The method of claim 5, further comprising:

(e) culturing the hybrid cells so as to express the antibody; and

(f) recovering the antibody from the hybrid cell culture.

7. The method of claim 5, wherein said nucleotide sequence is obtained from a B-cell or a hybridoma cell, wherein said B-cell or hybridoma cell produce an antibody.

8. The method of claim 5, wherein the first cell expresses an irrelevant light chain and expresses the desired heavy chain prior to fusion with the second cell.

9. The method of claim 5, wherein expression of the desired heavy chain by the first cell is determined by ELISA analysis of lysate from the first cell.

10. The method of claim 5, wherein the antibody is expressed only after fusion of said first and second cells.

11. The method of claim 5, wherein the first cell expressing the desired heavy chain is further selected for one or more desirable characteristics.

12. The method of claim 5, wherein both the second cell expressing the desired light chain and the first cell expressing the desired heavy chain are each further selected for one or more desirable characteristics.

13. The method of claim 12, wherein said desirable characteristic is selected from the group consisting of high production rate of the heavy chain and high production rate of light chain.

14. A method for producing an antibody, said method comprising:

(a) introducing a nucleotide sequence encoding a desired heavy chain into a first cell, wherein the first cell expresses an irrelevant light chain;

(b) introducing a nucleotide sequence encoding a desired light chain into a second cell; and

(c) fusing the first and second cells to form a hybrid cell, wherein the hybrid cell expresses the antibody.

15. The method of claim 14, further comprising:

(e) culturing the hybrid cells so as to express the antibody; and

(f) recovering the antibody .from the hybrid cell culture.

16. The method of claim 14, wherein said irrelevant light chain is present in an episomal vector.

17. A multi-component protein produced by the method of claim 1.

18. An antibody produced by the method of claim 5.

19. A antibody produced by the method of claim 14.

20. A method for screening for successful fusion of a first cell containing a first nucleotide sequence encoding a desired antibody heavy chain and a second cell containing a second nucleotide sequence encoding a desired antibody light chain, comprising:

including a nucleotide sequence encoding a first marker gene in the first cell;

including a nucleotide sequence encoding a second marker gene in the second cell;

fusing the first cell and the second cell under fuseogenic conditions to produce a fused cell; and

assaying for the presence of the first and second marker genes in the fused cell, wherein detection of the presence of the first and second marker genes in the fused cell indicates a successful fusion.

21. The method of claim 20, wherein the first marker gene is independently selected from the group consisting of the hygromycin resistance gene, the neomycin resistance gene, the hypoxanthine phosphoribosyl transferase gene, the dihydrofolate reductase gene, and the LacZ reporter gene and the second marker gene is independently selected from the group consisting of the hygromycin resistance gene, the neomycin

resistance gene, the hypoxanthine phosphoribosyl transferase gene, the dihydrofolate reductase gene, and the LacZ reporter gene.

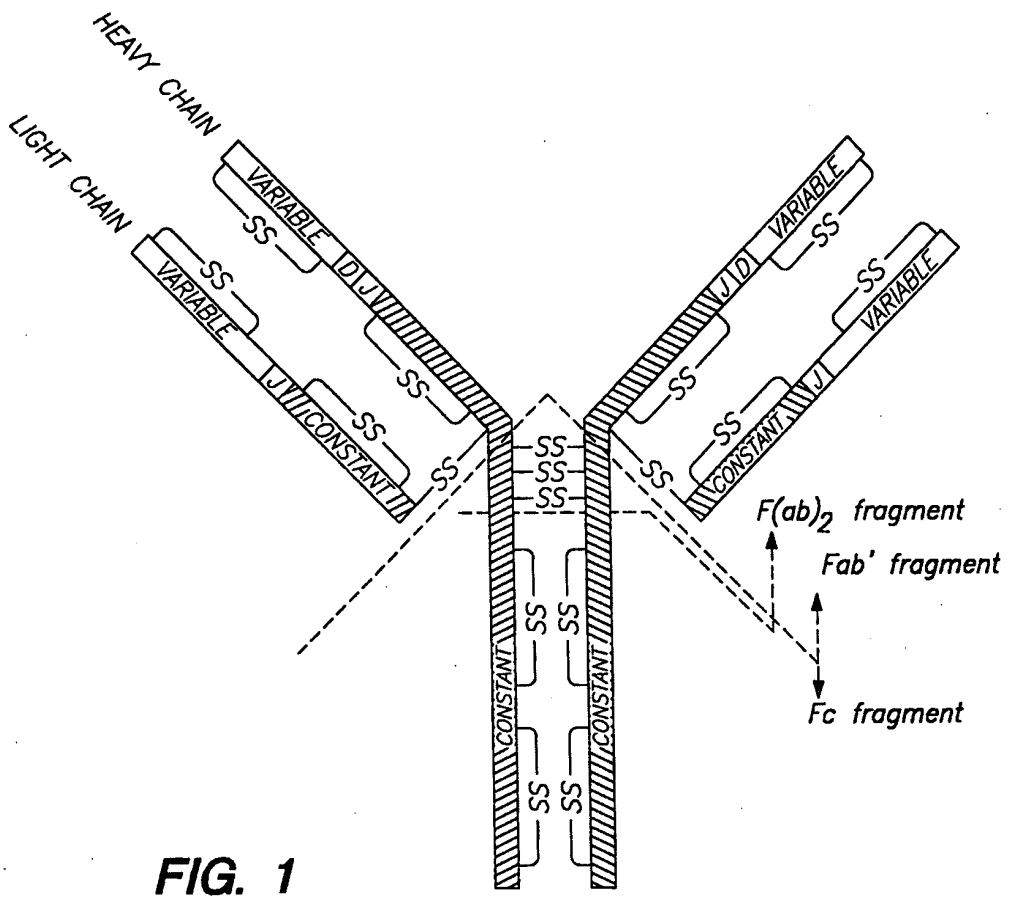


FIG. 1

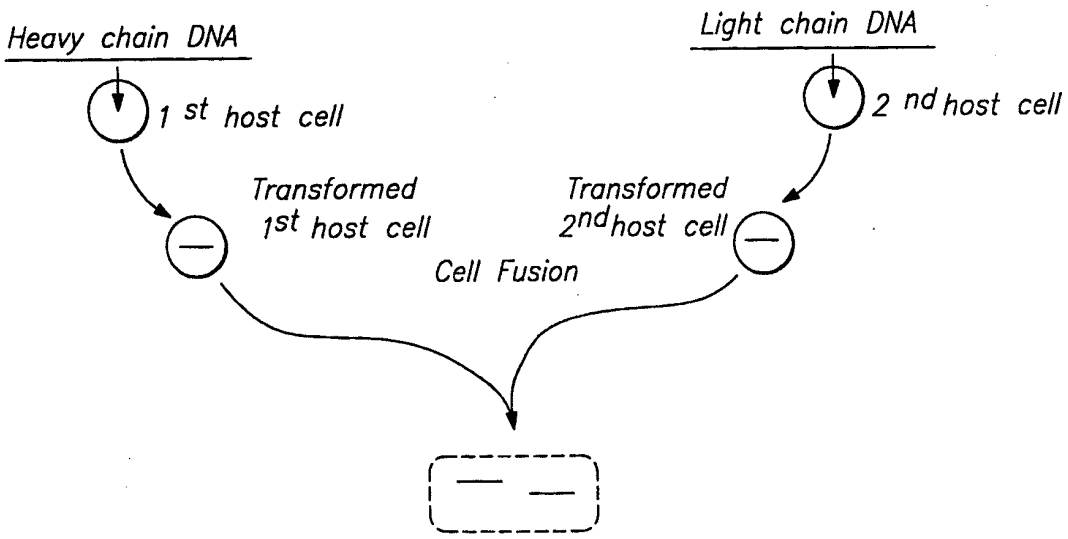


FIG. 2 Hybrid cell containing and expressing both heavy and light chain DNAs

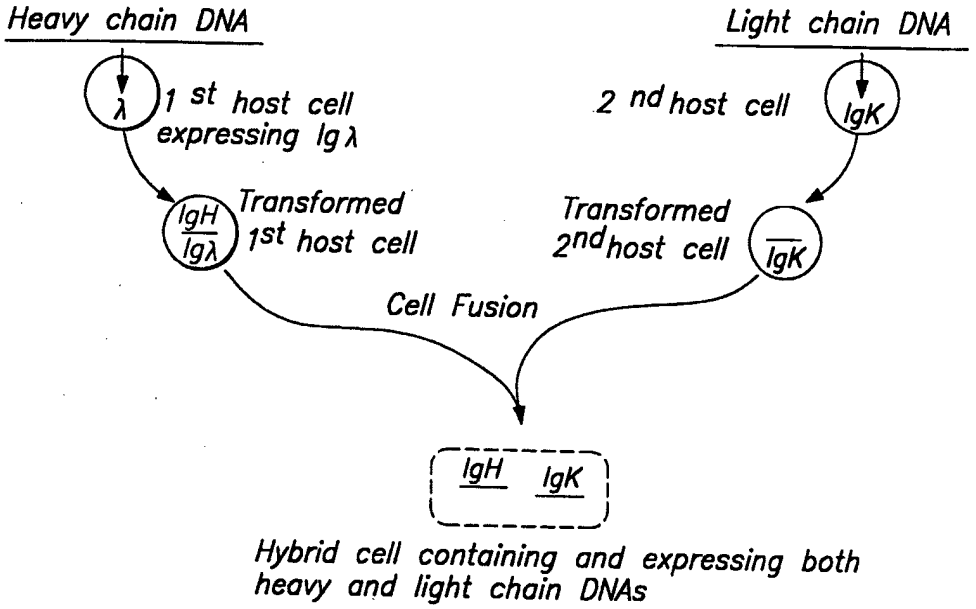

Heavy chain DNA
Light chain DNA
λ
1 st host cell expressing lg λ
2 nd host cell
lgK
lgH / lgλ
Transformed 1st host cell
Transformed 2nd host cell
lgK
Cell Fusion
lgH lgK
Hybrid cell containing and expressing both heavy and light chain DNAs

FIG. 3

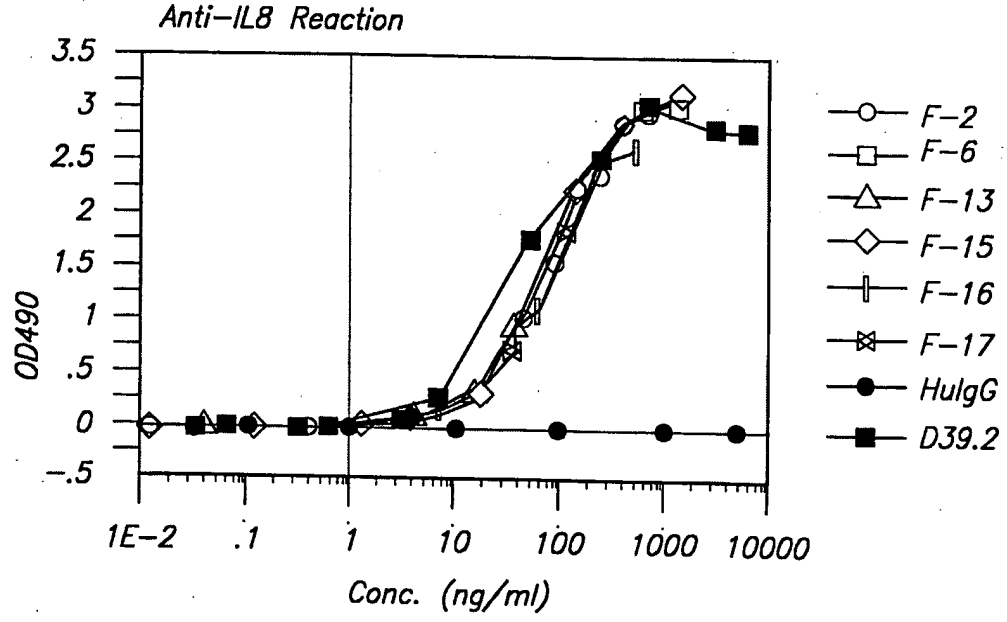

Anti–IL8 Reaction
3.5
3
2.5
2
1.5
1
.5
0
–.5
OD490
1E–2
.1
1
10
100
1000
10000
Conc. (ng/ml)
F–2
F–6
F–13
F–15
F–16
F–17
HulgG
D39.2

FIG. 7

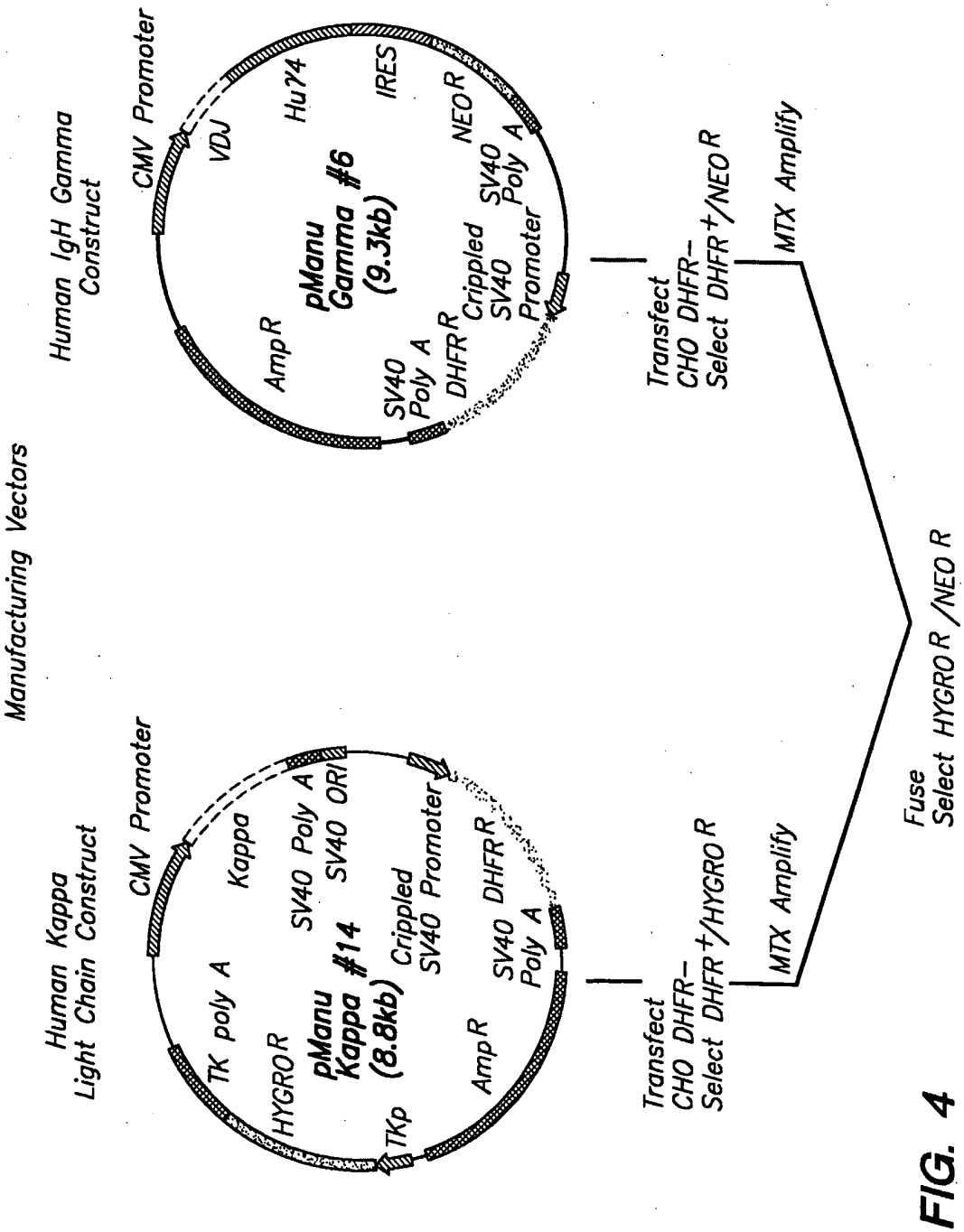

Manufacturing Vectors
Human Kappa Light Chain Construct
CMV Promoter
Kappa
SV40 Poly A
SV40 ORI
Crippled SV40 Promoter
SV40 Poly A
DHFR R
Amp R
TKp
HYGRO R
TK poly A
pManu Kappa #14 (8.8kb)
Transfect CHO DHFR−
Select DHFR+/HYGRO R
MTX Amplify
Human IgH Gamma Construct
CMV Promoter
VDJ
Hu γ4
IRES
NEO R
SV40 Poly A
Crippled SV40 Promoter
DHFR R
SV40 Poly A
Amp R
pManu Gamma #6 (9.3kb)
Transfect CHO DHFR−
Select DHFR+/NEO R
MTX Amplify
Fuse
Select HYGRO R /NEO R

FIG. 4

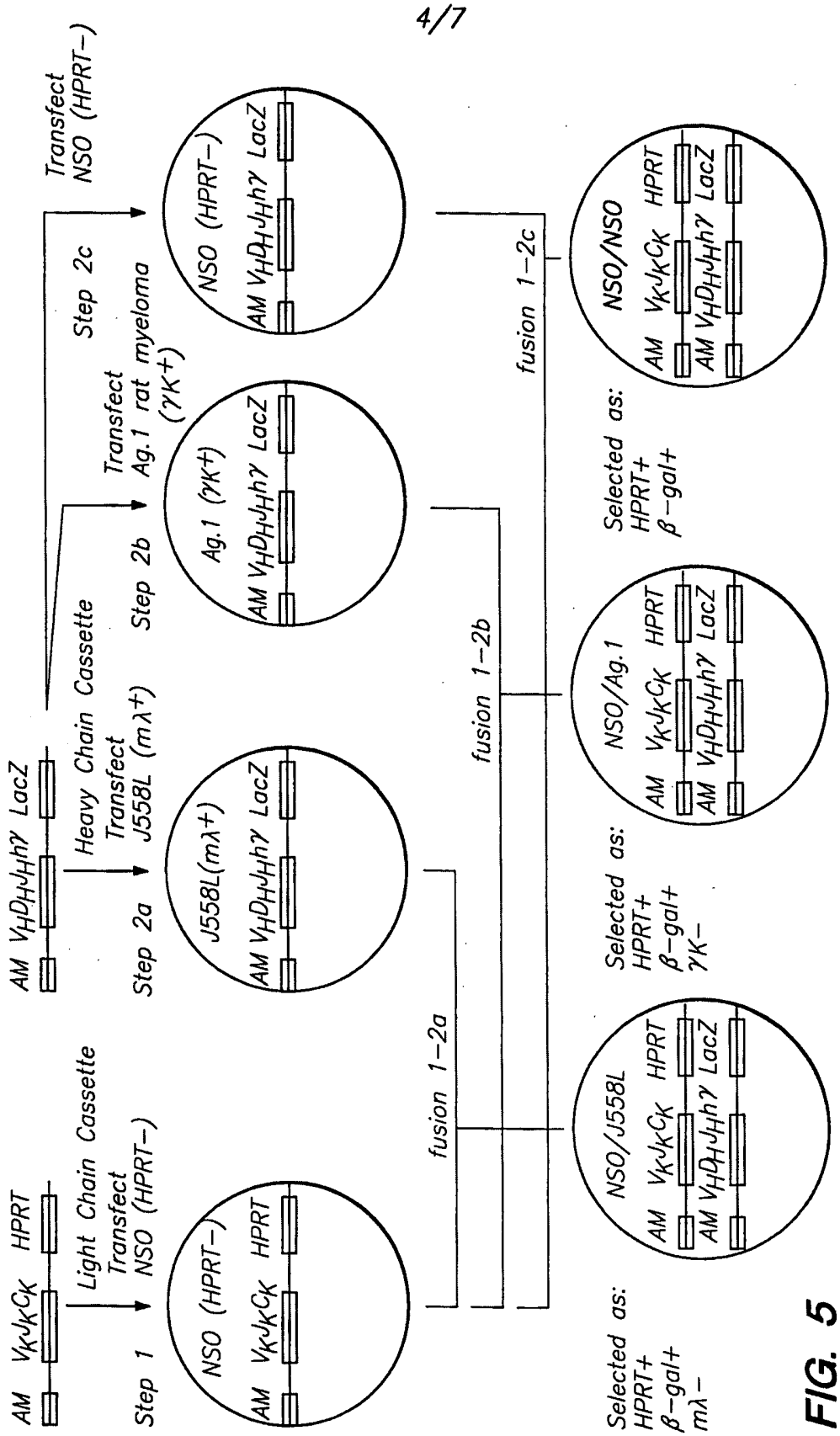

AM $V_K J_K C_K$ HPRT
Step 1
Light Chain Cassette Transfect NSO (HPRT−)
NSO (HPRT−)
AM $V_K J_K C_K$ HPRT
AM $V_H D_H J_H h\gamma$ LacZ
Step 2a
Heavy Chain Cassette Transfect J558L ($m\lambda^+$)
J558L($m\lambda^+$)
AM $V_H D_H J_H h\gamma$ LacZ
Step 2b
Transfect Ag.1 rat myeloma (γK^+)
Ag.1 (γK^+)
AM $V_H D_H J_H h\gamma$ LacZ
Step 2c
Transfect NSO (HPRT−)
NSO (HPRT−)
AM $V_H D_H J_H h\gamma$ LacZ
fusion 1−2a
fusion 1−2b
fusion 1−2c
Selected as: HPRT+ β−gal+ mλ−
NSO/J558L
AM $V_K J_K C_K$ HPRT
AM $V_H D_H J_H h\gamma$ LacZ
1−2a
Selected as: HPRT+ β−gal+ γK−
NSO/Ag.1
AM $V_K J_K C_K$ HPRT
AM $V_H D_H J_H h\gamma$ LacZ
1−2b
Selected as: HPRT+ β−gal+
NSO/NSO
AM $V_K J_K C_K$ HPRT
AM $V_H D_H J_H h\gamma$ LacZ
1−2c

FIG. 5

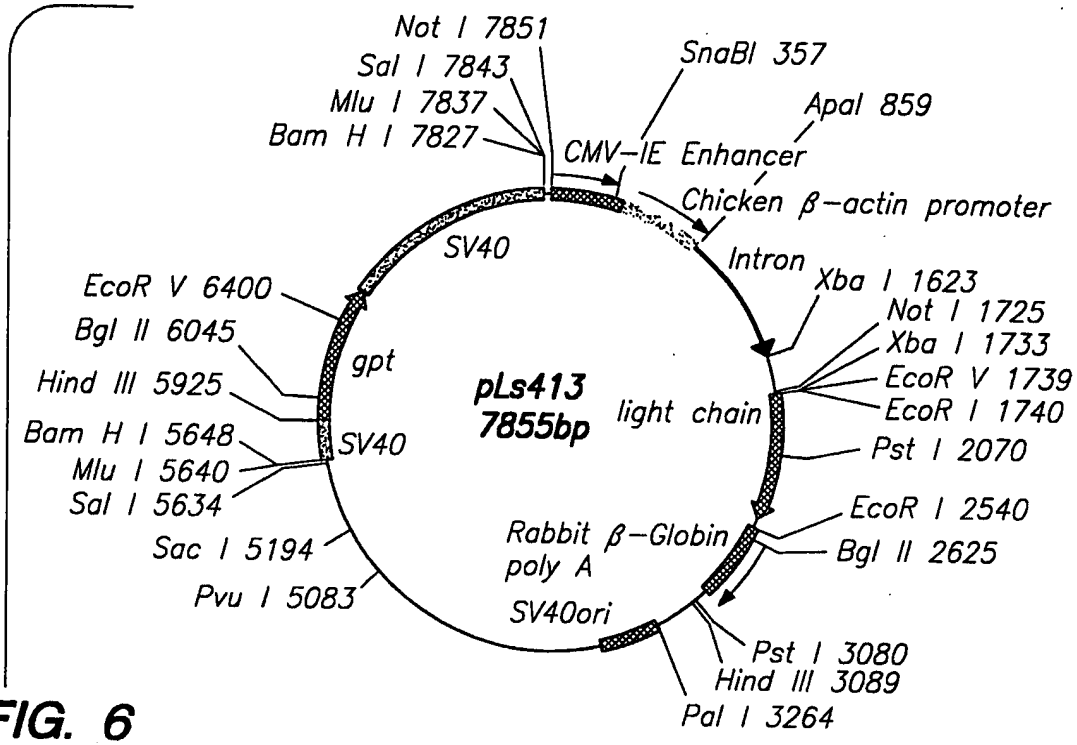
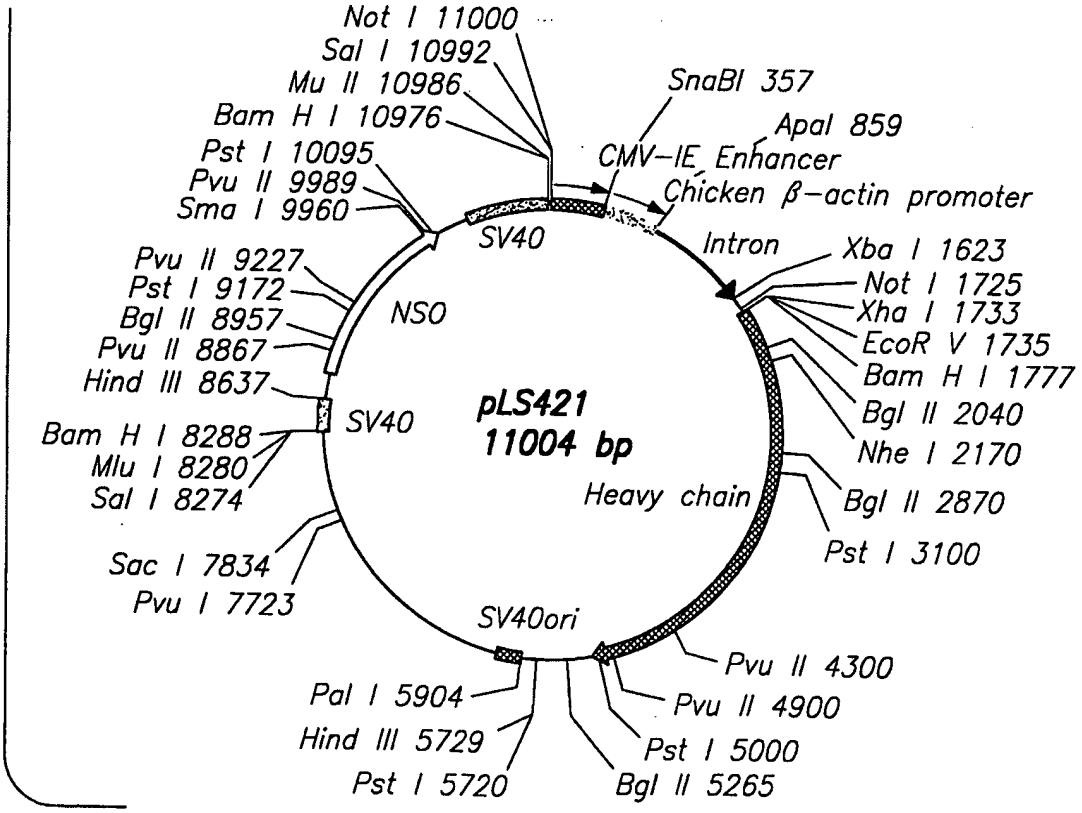
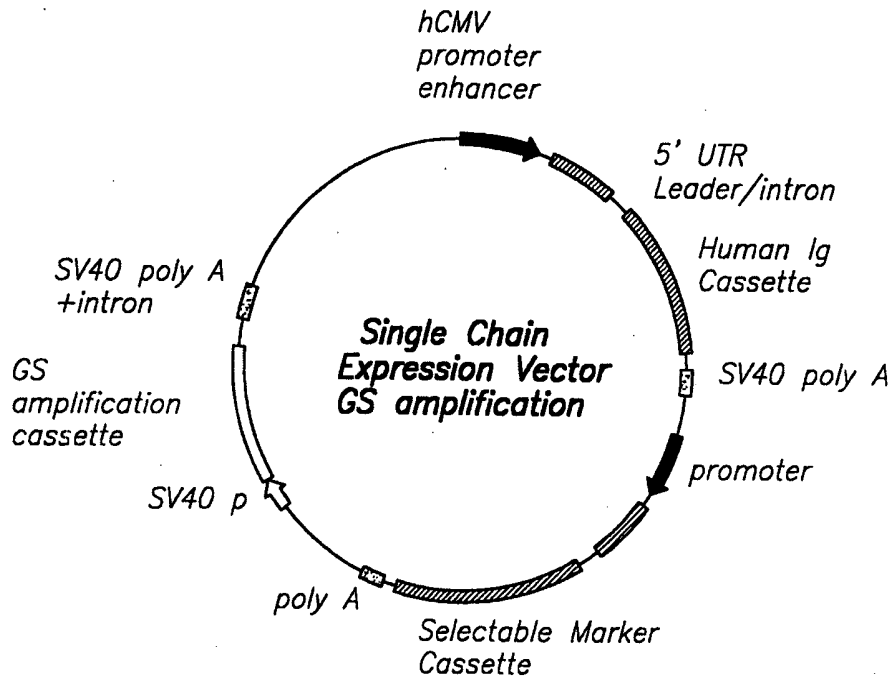

Not I 7851
Sal I 7843
Mlu I 7837
Bam H I 7827
SnaBI 357
ApaI 859
CMV-IE Enhancer
Chicken β-actin promoter
SV40
Intron
Xba I 1623
Not I 1725
Xba I 1733
EcoR V 1739
EcoR I 1740
Pst I 2070
EcoR I 2540
Bgl II 2625
Pst I 3080
Hind III 3089
PaI I 3264
EcoR V 6400
Bgl II 6045
Hind III 5925
Bam H I 5648
Mlu I 5640
Sal I 5634
Sac I 5194
Pvu I 5083
gpt
SV40
pLs413
7855bp
light chain
Rabbit β-Globin
poly A
SV40ori

FIG. 6


Not I 11000
Sal I 10992
Mu II 10986
Bam H I 10976
Pst I 10095
Pvu II 9989
Sma I 9960
SnaBI 357
ApaI 859
CMV-IE Enhancer
Chicken β-actin promoter
SV40
Intron
Xba I 1623
Not I 1725
Xha I 1733
EcoR V 1735
Bam H I 1777
Bgl II 2040
Nhe I 2170
Bgl II 2870
Pst I 3100
Pvu II 9227
Pst I 9172
Bgl II 8957
Pvu II 8867
Hind III 8637
Bam H I 8288
Mlu I 8280
Sal I 8274
Sac I 7834
Pvu I 7723
NSO
SV40
pLS421
11004 bp
Heavy chain
SV40ori
Pvu II 4300
Pvu II 4900
Pst I 5000
Bgl II 5265
Pal I 5904
Hind III 5729
Pst I 5720



Construct	cDNA "A"	Selectable Marker "B"
H/pEE 12.1	D1.1 VDJ Hu–Gamma 4	none
K/pEE 12.1	D1.1 Kappa	none
H/Pur/pEE 12.1	D1.1 VDJ Hu–Gamma 4	CMVp Puromycin SV40 Poly A
K/Hyg/pEE 12.1	D1.1 Kappa	TKp Hygromycin TK Poly A

FIG. 8

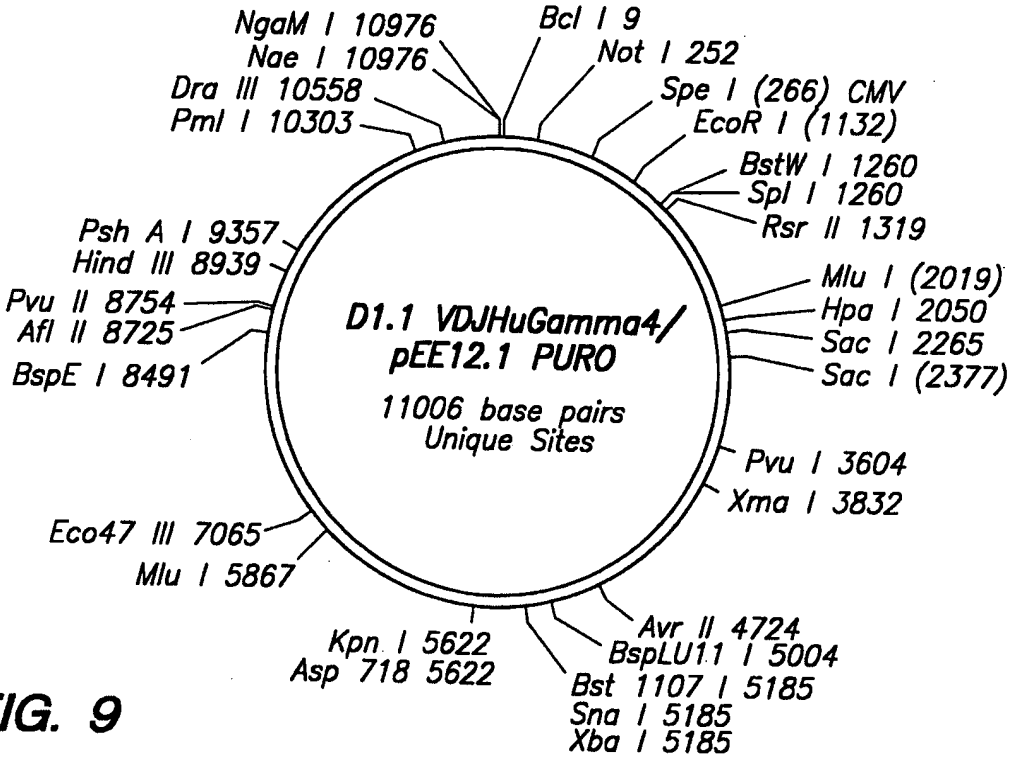

NgaM I 10976
Nae I 10976
Dra III 10558
PmI I 10303
Bcl I 9
Not I 252
Spe I (266) CMV
EcoR I (1132)
BstW I 1260
SpI I 1260
Rsr II 1319
Mlu I (2019)
Hpa I 2050
Sac I 2265
Sac I (2377)
Pvu I 3604
Xma I 3832
Avr II 4724
BspLU11 I 5004
Bst 1107 I 5185
Sna I 5185
Xba I 5185
Kpn I 5622
Asp 718 5622
Eco47 III 7065
Mlu I 5867
Psh A I 9357
Hind III 8939
Pvu II 8754
Afl II 8725
BspE I 8491
D1.1 VDJHuGamma4/
pEE12.1 PURO
11006 base pairs
Unique Sites

FIG. 9

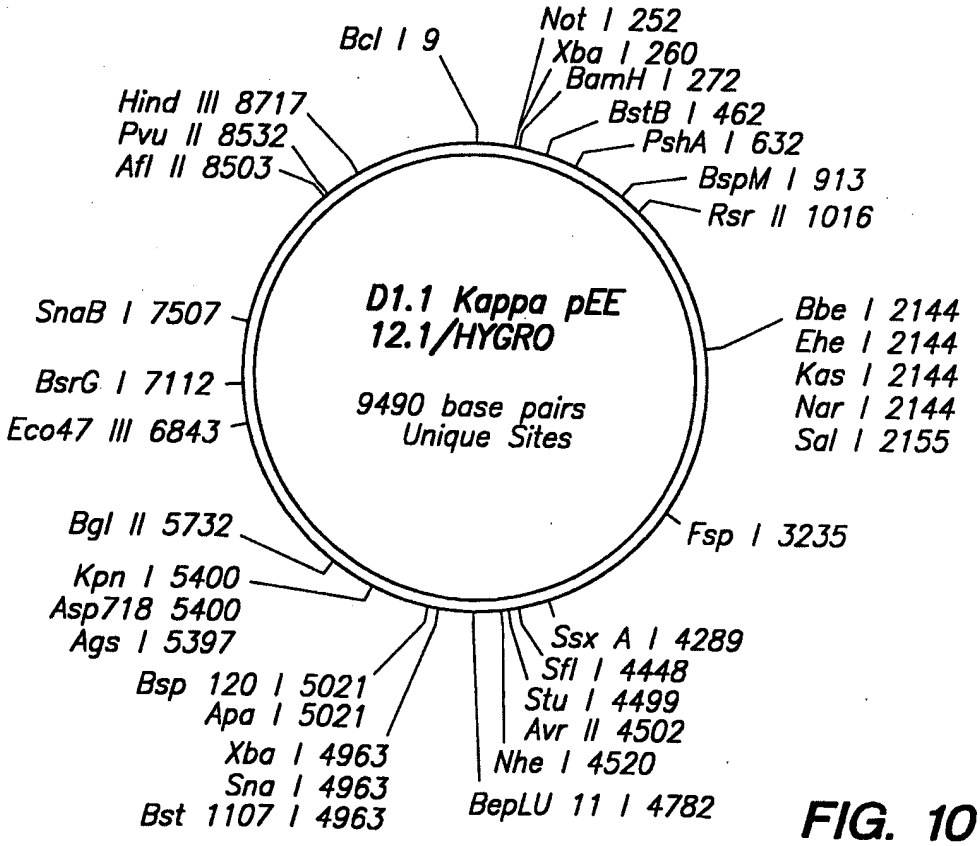

Not I 252
Xba I 260
BamH I 272
BstB I 462
PshA I 632
BspM I 913
Rsr II 1016
Bbe I 2144
Ehe I 2144
Kas I 2144
Nar I 2144
Sal I 2155
Fsp I 3235
Ssx A I 4289
SfI I 4448
Stu I 4499
Avr II 4502
Nhe I 4520
BepLU 11 I 4782
Bcl I 9
Hind III 8717
Pvu II 8532
Afl II 8503
SnaB I 7507
BsrG I 7112
Eco47 III 6843
Bgl II 5732
Kpn I 5400
Asp718 5400
Ags I 5397
Bsp 120 I 5021
Apa I 5021
Xba I 4963
Sna I 4963
Bst 1107 I 4963
D1.1 Kappa pEE
12.1/HYGRO
9490 base pairs
Unique Sites

FIG. 10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/18910

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12P 21/00; C12N 5/12, 15/00, 15/63; C07H 21/04
US CL :435/69.6, 70.1, 325, 326, 320.1; 536/23.1, 23.53

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.6, 70.1, 325, 326, 320.1; 536/23.1, 23.53

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

BIOSIS, EMBASE, BIOSIS, SCISEARCH, WPIDS
search terms: quadroma, cell fusion, multicomponent proteins

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Database on MEDLINE, Abstract No. 96075415, KRANENBORG et al., 'Development and characterization of anti-renal cell carcinoma x antichelate bispecific monoclonal antibodies for two-phase targeting of renal cell carcinoma', abstract, Cancer Research. 01 December 1995, Vol. 55, No. 23, 5864S-5867S, see entire abstract.	1-21
Y	Database on MEDLINE, Abstract No. 96085233, CAO et al., 'A rapid non-selective method to generate quadromas by microelctrofusion', abstract, J. Immunol. Methods. 16 November 1995, Vol. 187, No. 1, pages 1-7, see entire abstract.	1-21
Y	Database on EMBASE, Abstract No. 93278147, SALAZAR-KISH et al., 'Comparison of a quadroma and its parent hybridomas in fed batch culture', abstract, J. Biotechnology. 1993, Vol. 30, No. 3, pages 351-365, see entire abstract.	1-21

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
17 DECEMBER 1997	27 JAN 1998

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	GEETHA P. BANSAL
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/18910

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Database on MEDLINE, Abstract No. 92147173, BOS et al., 'Enhanced transfection of a bacterial plasmid into hybridoma cells by electroporation: Application for the selection of hybrid hybridoma (quadroma cell) lines', abstract, Hybridoma. February 1992, Vol. 11, No. 1, pages 41-51, see entire abstract.	1-21